Bacterial Analyses by Molecular Methods: Proposed Task 3.9

1.0 Introduction and Objectives

A potential key marker of contamination from chicken litter is the presence of selected bacteria known to originate from the litter. If it can be shown that these bacteria are present in the litter, the soil where the litter is applied, the nearby streams, and the downstream lakes, but not in pristine (noncontaminated) soils/water or in feces from other sources, then this will provide direct evidence that these areas in the watershed are impacted by litter disposal. A suite of molecular methods based on bacterial DNA analysis is proposed to pursue this objective. All of these methods rely on polymerase chain reaction (PCR) for amplification of DNA from samples to improve detection capabilities. Detailed methods for all of the techniques are provided at the end of this proposal (Attachment). Integration of the results obtained using the techniques described in the following tasks should provide a definitive identification and comprehensive understanding of how bacteria from the chicken litter are being transported and/or are propagating throughout the watershed. Unimpacted soils and feces from other sources will be used as controls to demonstrate whether selected bacteria of interest are unique to media impacted by the litter.

The following sections are divided into distinct tasks. At the end of tasks 1a and 2, an evaluation will be conducted to determine if the remaining tasks should be completed.

2.0 Task 1 – Identifying the Types and Abundance of Bacterial DNA (T-RFLP) and the Consistency of Sampling and Analyses

The first molecular technique is called terminal restriction fragment length polymorphism (T-RFLP). All of the bacterial DNA amplified by PCR is digested with an enzyme that cuts the DNA strands into different size fragments whose length is dependent upon the DNA sequence. Therefore, it is assumed that each fragment length represents a single species of bacteria. A histogram representing the microbial community structure is created that shows the relative abundance of different fragment lengths (i.e., different species). Applying T-RFLP to the samples collected will facilitate a rapid screening that will reveal whether particular fragment lengths that occur consistently in the litter are also prevalent in impacted soil and water samples, and are absent in unimpacted samples.

Subtask 1a - To determine which fragments are consistently found, replicate samples will be analyzed. In particular, five splits of two composite litter samples (10 samples)

and five splits of two composite soil samples (10 samples) will be analyzed using T-RFLP techniques. These analyses will help determine if major fragments are consistently found. If a subset of fragments cannot be consistently found in approximately 50 percent or more of the samples, evaluations will be conducted to determine if modifications to the remaining tasks should be made or if the additional tasks should not be performed.

Subtask 1b – If task 1a is completely successfully and consistent results are found, and if one or more of the fragments identified is unique based on Task 2, T-RFLP will be conducted on additional samples collected as part of Task 3.6, Soil and Litter/Manure Sampling. In particular, 15 litter samples, 40 soil samples from litter application fields and 20 control soils will be analyzed. In addition, 5 duplicates and 5 blanks will be analyzed.

3.0 Task 2 – Identifying the Types and Abundance of Bacterial DNA (T-RFLP) in Other Potential Source Materials

If consistent fragments are identified from chicken litter and associated soils, the second important step is to evaluate how unique the fragments are compared to other potential sources of bacteria. In particular, fecal material from the following sources will also be evaluated using T-RFLP techniques:

- Beef Cattle: During collection of the soil samples as part of Task 3.6, Soil and Litter/Manure Sampling, samples of cattle feces will also be collected. In particular, five individual samples from the fields of five different growers will be collected. These 25 samples will be analyzed using T-RFLP techniques.

- Dairy Cattle: Clean out slurry will be collected from one milking barn. Two samples will be collected and analyzed using T-RFLP techniques.

- Swine: Clean out slurry will be collected from two swine facilities. Two samples from each of the two facilities (four total samples) will be collected and analyzed using T-RFLP techniques.

- Duck and geese: Individual samples will be collected of duck and geese feces. At least three landing areas (e.g., wildlife areas) will be identified for collection if possible. At each area, five samples will be collected for a total of 15 samples. These samples will be analyzed using T-RFLP techniques.

- Humans: Septic tanks and/or septic tank clean out companies will be identified. Two samples will be collected from three different trucks that pump out the septic tanks (six total samples). These samples will be analyzed using T-RFLP techniques.

Once the analyses are completed, evaluations will be conducted to determine if a unique set of fragments are associated with the chicken litter. If a unique set cannot be identified, evaluations will be performed to determine if modifications to the remaining tasks should be made or if the additional tasks should not be performed. If unique fragments are determined, these fragments will be targeted for identification through the generation of clone libraries (task 3 below).

4.0 Task 3 - Identifying the Specific Bacterial DNA (Development of the DNA Clone Libraries)

The construction of the "clone libraries" that will be used to identify the most abundant and unique bacteria that appear to be moving from the litter into impacted soils and water comprises the second molecular technique. Clone libraries allow the separation of individual DNA sequences from the community DNA initially extracted from the samples (see detailed methods in the Attachment). Once separated, these sequences can then be determined and compared to bacterial DNA databases to establish the identity of the bacteria, or at least of the most closely related species in the database. Further, each of the individual DNA sequences thought to be important in the clone library is then analyzed using T-RFLP to determine the fragment length it generates. This information allows the key fragment lengths from the community T-RFLP analysis to be identified.

In the event that potential key indicator DNA fragment lengths appear in the control samples, clone libraries will be constructed and sequencing will be performed on both litter and control samples. The DNA sequences for the potential indicators in the litter will be compared to DNA sequences of bacteria producing the same fragment length derived from humans, cows and ducks to determine whether the sequences are unique to the chicken litter. DNA clone libraries will be developed on five of the litter samples collected as part of Task 3.6, Soil and Litter/Manure Sampling.

5.0 Task 4 – Validation of Quantitative Bacterial DNA Analysis (Q-PCR)

The third molecular technique is called quantitative PCR (Q-PCR). This tool facilitates extremely sensitive detection of a particular species of bacteria (very low detection limits). Once it is determined that one or more species present in the litter are of particular interest, Q-PCR can be used on samples to detect that species in very low concentrations, and to quantify the number of cells present in the sample. Besides definitively identifying a specific bacterium, the Q-PCR can be used to determine how the concentration of a particular bacterium changes from the litter to the environment (streams, sediments, lakes). It is assumed that Q-PCR protocols have already been developed for some of the more prominent species, so only minimal costs for protocol optimization are included in this estimate.

Q-PCR will be performed on 20 samples, 2 duplicates and 2 blanks to validate the analytical method. If it is determined that the method is effective, it can be applied to a full spectrum of samples.

6.0 Task 5 – Validation of Standard Bacterial DNA Analyses (Standard PCR)

In addition to Q-PCR, standard PCR methods will be evaluated and validated. Standard PCR only identifies the presence or absence of the specific bacterium in the sample tested versus quantifying the concentration of the specific bacterium (Q-PCR). Standard PCR is an important screening tool that is less expensive than Q-PCR, but still quite sensitive. It can provide definitive identification of specific bacteria (which can be confirmed by sequencing the amplified DNA), and therefore a definitive link to the litter. It is assumed that standard PCR primers have already been developed for some of the more prominent species, so only minimal costs for protocol optimization are included in this estimate.

Standard-PCR will be performed on 20 samples, 2 duplicates and 2 blanks to validate the analytical method. If it is determined that the method is effective, it can be applied to a full spectrum of samples.

7.0 Task 6 – Report Preparation

A report will be prepared summarizing the results of the above tasks. In particular, recommendations will be made concerning the use of the molecular methods in identifying and quantifying specific bacteria associated with chicken litter in environmental samples collected from streams, sediments, and lakes in the Illinois River watershed. If proven to be a valid and appropriate method, we anticipate that environmental samples can be analyzed for up to four specific bacteria by Q-PCR (quantitative analyses) for approximately $300/sample and by standard PCR (identification only of one bacterium) for approximately $100/sample. These costs include extraction of the DNA (see task 8.0). The report will refine these costs and make recommendations concerning which samples should be analyzed by which method.

8.0 Costs and Schedule

The estimated costs for the above task are provided in Table 8-1. Once the litter and soil samples are collected, the above tasks should be completed in six to eight weeks. This means that other environmental samples being collected during this time could be prepared for molecular analyses (Q-PCR or standard PCR) by extracting the molecular DNA from the sample. Extraction of the DNA costs $56/sample. This DNA extract can be held frozen for up to one year before analysis.

Table 8-1: Estimated Costs					
Activity	**CDM Labor (hr)**	**CDM Labor ($)**	**CDM ODCs ($)**	**Laboratory**	**TOTAL ($)**
Task 1a - T-RFLP, Consistency	20	$4,700	$100	$5,000	$9,800
Task 1b - T-RFLP, Chicken	30	$7,050	$0	$21,500	$28,550
Task 2 – T-RFLP, Other Sources	130	$18,350	$1,700	$13,000	$33,050
Task 3 - Clone Libraries	20	$4,700	$100	$9,125	$13,925
Task 4 - Q-PCR	20	$4,700	$400	$13,145	$18,245
Task 5 - Standard PCR	20	$4,700	$0	$5,574	$10,274
Task 6 - Reports	90	$15,850	$300	$7,490	$23,640
Totals	**330**	**$60,050**	**$2,600**	**$74,834**	**$137,484**

Attachment – Analytical Methods

Methods

The methods described below have been generalized from Macbeth et al. (2004).

Extraction of nucleic acids from water samples.

Community DNA from soils and water will be extracted with the FastDNA SPIN kit for soil (Bio 101) according to the manufacturer's instructions, eluted in nuclease-free water (50 µl) and stored at -20°C.

16S ribosomal DNA (rDNA)-targeted PCR.

PCR is used to amplify nearly full-length 16S rRNA genes from *Bacteria*. Each 25-µl PCR mixture includes 0.2 mg of molecular-grade bovine serum albumin (Sigma Chemicals) ml^{-1}, 1X PCR buffer (Promega), 1.5 mM MgCl2, 0.5 µM (each) forward and reverse primer (Invitrogen), 1 U of *Taq* DNA polymerase (Promega), 0.2 mM (each) deoxynucleoside triphosphate (Invitrogen), 1 µl of template DNA, and molecular-grade water (Promega). Universal primer 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') is used in conjunction with eubacterial primer 8F (5'-AGA GTT TGA TCC TGG CTC AG-3'), targeting the 16S rRNA genes of *Bacteria*. Amplification is typically performed on a Perkin-Elmer model 9600 thermocycler by the following regimen: 95°C (5 min), followed by 25 cycles of 95°C (1 min), 53.5°C (1 min), and 72°C (1 min). The reaction is finished with an additional 7 min at 72°C. PCR products are examined in a 1.2% agarose gel prior to clone library construction to confirm specificity of the amplification reactions. PCR for T-RFLP employs primers 8F, modified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM; Invitrogen), and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). Each PCR (25-µl mixture) is performed in triplicate by the PCR and thermocycler protocols described above.

Construction of bacterial 16S rDNA clone libraries.

Nearly full-length 16S rDNA PCR products amplified from the samples are purified (QIAquick PCR purification kits; QIAGEN) and cloned into the p-GemT Easy vector (Promega) according to the manufacturer's instructions. Transformants are selected with standard blue-white screening on plates of S-Gal (3,4-cyclohexenoesculetin-β-D-alactopyranoside; Sigma) with ampicillin. Plasmids are purified from selected transformants. Plasmid DNA is extracted and purified from cultures of each clone grown in 1 ml of TPYNG medium containing ampicillin with the QIAprep Spin Miniprep kit (QIAGEN).

T-RFLP analysis.

The triplicate PCR products generated from each DNA sample (combined triplicate extractions) are combined and purified with the QIAquick PCR purification kits (QIAGEN). PCR product concentration is estimated by measurement of absorbed UV light at a λ of 260 nm. Approximately 200 ng of PCR product is digested with MspI (1

U) (New England BioLabs; 37°C, 3 h). The digested fragments are purified by ethanol precipitation, and the DNA pellet is resuspended in 10 µl of DNase-free water. Samples are denatured by heating to 95°C for 3 min followed by submersion in an ice bath. The denatured DNA (2 µl), along with the internal standard Rox 1000 (Applied Biosystems), was loaded onto a model 3100 DNA sequencer (Applied Biosystems). The resulting data were analyzed with Genescan (Applied Biosystems).

Each PCR product is digested once, and each digest is run in triplicate on the DNA sequencer to generate replicate T-RFLP profiles of the samples. These replicates are aligned manually, and composite profiles are generated. Methods described by Dunbar et al. (2000, 2001) are used to ensure that the fragments are real and not artifacts of the method. Therefore, only those fragments exhibiting high reproducibility are evaluated. Briefly, terminal restriction fragments (T-RFs) in different replicates that differ by less than 0.5 bp are considered identical and assigned the average size and peak heights of the fragments making up the composite T-RF. Any T-RF not present in all of the replicate profiles is discarded. Likewise, any T-RF within a composite profile with peak height averaging ≤25 fluorescence units was also discarded. The sum of all T-RF peak heights ≥25 florescence units is used as an indicator of the total DNA quantity represented, and peak heights are normalized to the replicate with the lowest total fluorescence.

T-RF peaks represented in the samples will be indirectly identified by digesting individual clones from 16S rDNA libraries and identifying their corresponding T-RFs. The PCR and T-RFLP protocols are the same as those described above except that 25 ng of plasmid is used as the PCR template. Although each clone typically generates a single T-RF, multiple clones might generate the same size T-RF. Theoretical T-RF sizes can also be determined "in silico" by locating restriction sites within the clones having known DNA sequences.

Q-PCR Analysis.

It is assumed that Q-PCR primers for the bacteria of interest have already been developed. If not, they can be designed, but that process is not accounted for in this proposal. Amplification and detection of DNA by Q-PCR can be performed with the Opticon Sequence Detection System (MJ Research). Q-PCR analyses will be performed in accordance with published protocols for the species of interest.

References

Dunbar, J., L. O. Ticknor, and C. R. Kuske. 2000. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl. Environ. Microbiol. 66:2943–2950.

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Macbeth, T. W., D. E. Cummings, S. Spring, L. M. Petzke, and K. S. Sorenson. 2004. Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep fractured basalt aquifer and comparison to a derivative laboratory culture. Appl. Environ. Microbiol. 70:7329-7341.